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OSTEON MIMETIC SCAFFOLDING

By

Janay Clytus

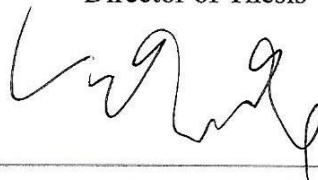
Submitted in Partial Fulfillment
of the Requirements for
Graduation with Honors from the
South Carolina Honors College

May 2018

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Thesis Summary

This thesis investigates the process of bone formation in order to create an artificial scaffold to promote bone regeneration. The research examines current practices that are used to reduce tissue rejection and optimize bone healing. An artificial scaffold can be successfully created using calcium phosphate ceramics with positive mechanical characteristics for cell infiltration and cell viability.

Abstract

The purpose of this research is to provide an alternative to naturally derived bone grafts. There is a gap in the supply of donors and the demand of bone tissue. Artificial scaffold creation can work as an implant and decrease the shortage of bone grafts and increase the range of injuries that can be repaired. Current research focuses on optimizing mechanical properties such as porosity, improving vascularization using cells, and generating osteoconductivity. For osteodifferentiation, mesenchymal stem cells (MSCs) can differentiate into mesodermal lineages such as chondrocytes, osteoblasts, adipocytes, and tenocytes by supplementing cultures with lineage-specific soluble factors (Marchetti). Co-culturing ECFCs with MSCs has is known to promote vasculogenesis (Shafiee). The calcium content effect on the vasculogenic potential of ECFCs has not been thoroughly studied and would be a novel direction for further work. The Ca^{2+} ion is known to play a role in cell signaling, such as in the Wnt signaling pathway. Increased Ca^{2+} concentration could increase the cellular proliferation of ECFCs and cause upregulated expression of VEGFA protein-coding gene. Including the hydroxyapatite (HA) and tricalcium phosphate (TCP) scaffold could potentially increase the proliferative ability of the stem cells and enhance the healing process for the bone repair. Ensuring proper pore structure and matrix structure also ensures cell viability and optimal mechanical demands such as for load

bearing applications. The scaffold is a suitable material for cell adhesion, and the scaffold can withstand degradation by macrophages therefore in the body the scaffold will be able to last for the typical amount of time it takes for the fracture to heal. For 100% degradation this would be estimated to take approximately 104 days.

Introduction

Tissue engineering has been one of the most challenging fields of research during recent years. Conventional methods for replacing vascular tissue have many shortcomings and may not work for some patients. The U.S. Department of Health and Human Services reports that someone is added to the transplant waiting list every 10 minutes, and as a result 22 people die each day while waiting for a transplant (Anderson-Shaw). The amount of donors does not match the amount people waiting to be organ recipients. This research focuses on mimicking the osteon microstructure in order to provide a biocompatible and osteoconductive material for bone repair. Techniques for bone implants currently focus on utilizing autografts, allografts, xenografts, or metallic and plastic implants (Shayesteh, Amini, Shibuya). More than half a million patients receive bone defect repairs annually in the United States with a cost greater than \$2.5 billion (Amini). Autografts take bone tissue from the patient in order to fix the area in need (Amini). The drawbacks include a high risk of bleeding, scarring, donor site deformity, inflammation, infection, and chronic pain (Amini, Polo-Corrales) They require a second operation and can be very expensive (Amini, Polo-Corrales). Allografts are a bone-grafting technique where donor bone tissue, often from a cadaver, is transplanted to the patient (Amini). They eliminate the need for a second procedure; however, allografts have a risk of immune reactions and infection transmission. Osteoinductive properties are reduced in an allograft and there is no cellular component, so the graft is slow to integrate with the host and can lead to tissue rejection (Amini,

Shibuya). Immunosuppressive therapy is used to counteract tissue rejection, but it has both beneficial and detrimental effects on the transplant recipient. The negative effects of the therapy arise from the nonspecificity of the drugs administered and result in an increased risk for infection (Israeli). Xenografts have higher antigenicity since they come from a nonhuman species and have reduced osteoinductive properties due to its need for more sterile processing (Shibuya). Hyperacute rejection is exacerbated in xenografts because complement-regulatory proteins such as CD59, DAF (CD55), and MCP (CD46) work less efficiently across species (Janeway). Additionally, metallic implants have made advances, but tend to activate immune responses and have mechanical failures in the long term.¹ Typically, doctors also graft veins from other parts of the person's body, but in one-third of all cases patients do not have healthy veins to graft from other locations (Amini).

The current state of tissue transplantation still needs improvement, due to a large shortage in available tissue donors and major complications that can arise from currently widely used techniques. Bones accomplish a range functions including locomotion, protection of our internal organs, housing biological elements required for hematopoiesis, trapping dangerous metals, maintaining homeostasis of key electrolytes, and responding to metabolic, physical and endocrine stimuli (Amini). Another importance is vascularization. Bone vascularization is key to creating implants that will not be rejected and fail in the body (Lan-Levengood, Auger). Using the above considerations, this study will synthesize a biomimetic scaffold. By mimicking the osteon microstructure successfully, we will accomplish increased porosity for the microvasculature to develop in. Porous 3D scaffolds promote new tissue formation by providing a surface that promotes attachment and cellular migration which is necessary for proliferation and differentiation (Boccaccini). For additional enhancement, endothelial colony forming cells

(ECFCs) have significant potential for expedited neovascularization (Lan-Levengood, Amini, Cosson). A study found that activation of the Wnt pathway improved the capacity of ECFCs to form capillary-like networks in vitro and in vivo (Fraineau).

Bone disorders worldwide are expected to double in 2020, especially with the growth of an aging population (such as the baby boomers) and increased obesity (Amini). To prepare for this rise more successful synthetic grafts need to be created. Furthermore, creating improved grafts will lead to less return patients due to complications, which will additionally lessen the burden on medical staff. The combination of hydrogel + ECFCs + hydroxyapatite + TCP with controlled pore distribution combines the optimal properties for successful bone constructs (due to its vascular potential, natural-mimicking, live cellular components and retention time) and the combination shows high potential for creation of a highly adaptable and cutting edge synthetic bone implant (Gómez, Chen, Zhang, Tampieri, Lachmann). This could also potentially help patients with Rickets related fractures (Braithwaite).

Advantages are that the scaffold could provide controlled and sustained drug delivery (Rahman). Also this calcium phosphate ceramic scaffold can be used for load bearing applications, unlike many conventional bone pastes. The scaffold also has a proper degradation rate of 104 days which is comparable to the amount of time fractures took to heal in a study. Hydroxyapatite (HA), the crystalline form of calcium phosphate, forms 60-70 per cent of bone tissues and provides increased mechanical strength (Sulaiman). Tricalcium phosphate serves as a rich source for calcium and phosphorus, which can be easily assimilated and absorbed. Beta-tricalcium phosphate is also biocompatible and creates a resorbable interlocking network promote healing (Sulaiman). The scaffold serves as an osteoconductive matrix. It will promote the formation of bone more an area void of scaffolding material (Albrektsson).

Developmental Processes that Govern Recovery

During the regeneration process the size of the damaged tissue creates a challenge for repairing defects (Boccaccini). Design challenges include fabrication of precisely and spatially patterned, highly porous scaffolds/matrixes to guide the overall shape of tissue growth and replacement. Tissue engineering has focused on reconstructing small defects (Boccaccini) . Today, thick tissue engineered scaffolds remain a challenge due to having limited diffusion of oxygen and nutrients to the interior part of the scaffolds which leads to improper tissue regeneration (Boccaccini, Yasar, Amini). Differences in regeneration methods of variable tissue types present another challenge to replicate the tissues synthetically. Each tissue type has its own regeneration process that needs certain components and signaling (including those signaling for recruiting other cell types such as macrophages to remove the dead cells and debris and then progenitors to be differentiated and help regeneration of the damaged tissue) [Zhu, Tedesco, Bishi, Raff, Rinkevich].

Material composition is also crucial for tissue engineered structures due to the need for biocompatibility for correct tissue regeneration. In order to overcome these design and fabrication challenges, research has been expanded to generation of scaffolds which have inbuilt micro and nanoscale fluidic channels that induce proper overall shape of regenerated tissues (Yasar,Amini).

Osteogenesis

The osteon is a laminar structure that has veins and arteries that bring nutrients or take away nutrients from the tissue and is the structural unit of cortical bone. The calcified bone tissue consists of three elements: cells, organic matrix and inorganic substances. These components are dependent upon each other. The cells are known to assemble the organic matrix and generate the

organic molecules that participate in the process of calcification (Ascenzi). Osteoblasts, osteocytes and osteoclasts are the cells present in the bone tissue. Osteoblasts synthesize collagen and are therefore considered bone forming cells. Osteocytes make up over 90% of all bone cells and are osteoblasts that become entrapped in the matrix that they produce. Osteoclasts are primarily responsible for bone resorption (Ascenzi). Osteocalcin is in the bone matrix and is assembled by osteoblast cells and shows calcium-binding characteristics that help its interaction with hydroxyapatite (Ascenzi). Osteocalcin close interaction with hydroxyapatite is hypothesized to inhibit growth of crystalline hydroxyapatite and to guide its shape and size (Ascenzi). After the collagen is laid down by the osteoblasts, the process of calcification starts and leads to the formation of a solid, stable, crystalline inorganic phase within the organic phase. Carbonated hydroxyapatite crystals generally parallel collagen bundles. Scaffolding can help to control geometry, which is significant because the geometric orientation can alter the bone's mechanical properties. This is similar to how the orientation of collagen fibrils is important because differences in the amount of space between fibers allows for different amounts of non-collagenous material to build up between collagenous layers (Ascenzi). MSCs are known to promote osteogenesis. MSCs differentiate into osteoblasts mediated in part by bone morphogenetic proteins (BMPs) signaling (Shakesheff). Osteopontin and Runx2 genes expression can be measured as a sign of cellular differentiation (Wang, L). Osteogenic differentiation of MSCs can also be measured by monitoring alkaline phosphatase (ALP) activity (Birmingham).

Chondrogenesis

Chondrogenesis is the process of collagen creation. Collagen is the precursor to bone formation. Fibroblasts produce collagen. Through a process called endochondral ossification,

there is aggregation and differentiation of mesenchymal cells, and proliferation, enlargement of tissue due to an increase in cell size, and death of chondrocytes (Cancedda). Precursor mesenchymal cells lead to chondrogenic and myogenic lineages that decide the differentiation of cartilage (Goldring). Differentiation of chondroprogenitors is characterized by the addition of cartilage matrix with collagens II, IX, and XI and aggrecan (Goldring). Bone formation starts in the content surrounding the hypertrophic cartilage core where blood vessels later grow into, then this is replaced by bone tissue and bone marrow (Cancedda).

Vasculogenesis

Vasculogenesis is the formation of new vascular networks from endothelial progenitor cells (angioblasts) and angiogenesis is the formation of new blood vessels from growth of nearby blood vessels (Fujita, Rouwkema). Embryonic, fetal, and postnatal stem cells as well as various endothelial progenitor cells can be a good cellular source for vascular tissue engineering (Tian). There are 3 different ways to develop vascular bioengineered tissues: incorporation of angiogenic factors, seeding endothelial cells with other cell types, and prevascularization. Studies have shown that retention of transplanted cells in the desired site is still problematic to prevascularize a tissue, consequently different materials such as scaffolds, matrigels, and membrane matrixes have been investigated as extracellular environments to overcome this problem (Kurita, Hanjaya-Putra). Hydrogels have been increasingly investigated as tool to keep seeded cells in-site and prevent ischemia (Hanjaya-Putra). PEG hydrogels are less degradable than other hydrogels without and enzymatically cleavable peptide. A single resilin-like polypeptide, RLP24, can be added to PEG hydrogels and creates a degradable, elastic, rapidly forming and rubber-like hydrogel; polypeptide incorporation would be useful for mechanically demanding tissue engineering applications, such as cardiovascular tissues (Linville). Protein-

PEG hydrogels comprising other proteins, such as collagen or fibrinogen, have been shown to improve biocompatibility and cell–matrix interaction (Linville). Hydrogel stiffness and chemical composition can change the levels of cell responsivity, cell migration, and cell adhesion. Trends show that adherent cells will migrate from soft to stiffer regions within a hydrogel which allow for artificial directing to induce desired synthetic biomaterials (Ahearne). Cell migration control is a main component behind creating the radial structures needed for vascular structures. The epithelial–mesenchymal transition process, changes in cell phenotype, extracellular matrix–cell interaction, cell–cell interaction, and soluble factor gradients contribute to cell migration processes and high cross-linking density can impede cell migration (Ahearne). A promising technique is using stem cells to have a lower chance of the body rejecting the tissue transplant (Bonde). Endothelial colony forming cells are a subset of endothelial progenitor cells (Lin, Prasain). ECFCs are rapidly recruited to the site of vascular injury or tissue ischemia after intravenous injection, where they initiate a vasculogenic response (Nutan). ECFC's can be encapsulated in a hydrogel environment and have been shown to promote neovascularization. ECFC also provide trophic support of MSCs (driven by PDGF-BB/PDGFR- β signaling). It was later found that photo-crosslinkable methacrylated gelatin (GelMA) hydrogels are compatible with ECFC-based vascular morphogenesis. GelMA hydrogels polymerize rapidly, which limits dissemination at the implantation site and eliminates the need for pre-polymerizing the constructs, and resulted in improved integration with the surrounding host tissue and better vascularity in injected constructs (Marchetti). These properties make GelMA hydrogels a better use than PEG based hydrogels. The scaffold pore size at 500 μ m which is large enough for red blood cells which have an average diameter of 7-8 μ m; at this size after vascular structures are produced in these pores there is also become subjected to an Fahraeus-Lindqvist effect will be

produced where blood viscosity is lowered at microvascular levels. There is a significant decrease in the apparent viscosity (μa) of blood between tube diameters from about 500 μm to 4–6 μm as diameter due to the the decrease in the tube hematocrit. Reducing Hct might be favorable in terms of increasing O(2) flux and pO(2,min), so developing microvasculature at using this pore size could help improve oxygen transfer (Kwon).

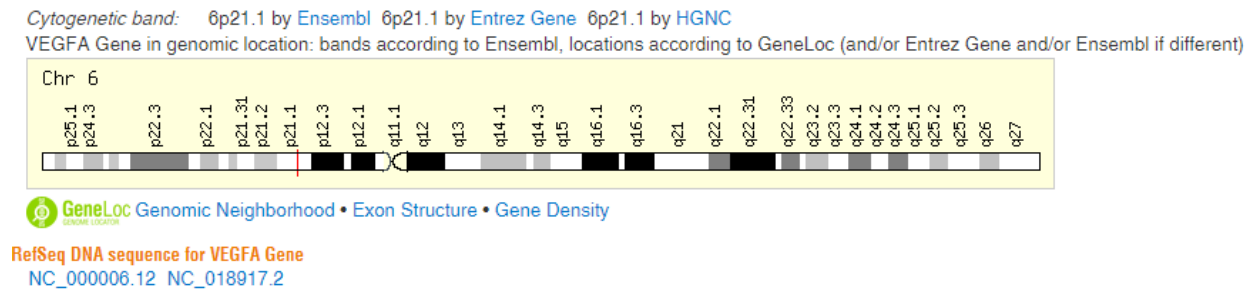


Figure 1. VEGFA Gene information (Weizmann, 2017).

Techniques to develop in-vitro vasculature

As mentioned before, faster rates of neo vascularization of implants, is a prerequisite for in vivo tissue regeneration. A challenge is that each kind of cell needs different kinds of nutrients and an appropriate niche in order to grow, proliferate, and differentiate. Thus the vital mechanism is the kind of biomaterial selected for developing vasculature. The kind and properties of the material and its behavior as 3D scaffold would directly affect vascularization process. For instance, in order to have a prevascularized bone scaffold, the 3D construct must support different cell types (Santos). For this aim several models have been analyzed for in vitro vasculogenesis and many 3D models were prepared and have been investigated.

For some applications, a 3D environment is difficult to form and maintain capillary like networks. Manipulation of complex 3D structures was investigated to optimize the environment to form these networks. The extravascular matrix and cellular environment significantly affects

vascular character (through biochemical signals that correspond to these components) along with microarchitecture, deformation, and mutability of the matrix (Gieni). Growth factors have crucial role in controlled vasculogenesis. Scaffolds have to be designed for controlled release of these growth factors. According to Kaul et al. such scaffolds can be divided into two main groups: scaffolds that have “single GF delivery” and those which have “multiple GF sustained delivery in a controlled manner” (Kaul). Naturally, the vascular smooth muscle cell synthesizes and organizes the extracellular matrix (ECM) in medium to large vessels (Xu). Under pathological conditions, vascular matrix proteins (fibrillar types I and III collagen, chondroitin sulfate and dermatan sulfate proteoglycans, fibronectin, and others) undergo proteolytic processing, yielding bioactive fragments to influence vascular wall matrix remodeling. Vascular cells also produce alternatively spliced variants that induce vascular cell production of different matrix proteins to interrupt matrix homeostasis, leading to increased blood vessel stiffness; vascular cell migration, proliferation, or death; or vascular wall leakage and rupture (Xu). The vascular wall ECM allows elastic recoil (Wagenseil). Blood vessels are exposed to radial, axial, and circumferential strain. This stretching pressure is felt by the circumferentially arranged cells through their interaction with ECM. There are local deformations in individual ECM fibers attached to membranes through transmembrane proteins such as integrins. The deformation causes conformational changes in ECM proteins and the integrins, triggering focal adhesion complex formation. Integrins are directly connected through the focal adhesions to cytoskeletal proteins that link all the way to the nucleus resulting in changes in nuclear shape and altered gene expression (Gieni). The average diametric size of capillaries is 8 microns (Freitas). In human tissues, average capillary density is ~600/mm (Freitas). Microvascularization is important to the overall survival of implanted engineered tissues.

In order to support dense tissues, micro fabrication is one of the methods to fabricate the constructs, as Kaul et al. mentioned, MEMS (micro electro mechanical system) is a special micro fabrication technique in respect to vasculogenesis (Kaul). Co-culturing is another in vitro model to mimic a natural growth environment for growth factors and cells. In hUVEC/hMSC co-cultures, human vascular endothelial growth factor (VEGF) expression was Upregulated after 3 weeks in vivo compared to MSC monocultures (Brennan). Spheroid culture is another method of propagating cells to create complex tissues (Fennema). Decellularizing cadaveric tissue was another approach to obtain a 3D scaffold.

Rapid prototyping

With the increasing portability, even to the size of a pen, of rapidly prototyping machines to build 3d constructs, this technique can be applied to enhance creation of vasculogenic hydrogels by incorporating the hydrogels within a matrix in order to have a backbone for structures that need preprogrammed shapes to properly form the target structures. Bioprinting is a well-known method to quickly form the matrixes necessary for vascularization. A patient-specific model of right ventricular outflow tract (RVOT) was captured by a 3D volume derived from CMR data, from which a physical phantom was rapidly prototyped and then used for physical insertion of a stent-graft for assessing patient's suitability for the device (Vismara). The rapid prototyping of RVOT model was well representative of the original anatomy and successful physical insertion of the novel graft indicated that the patient was suitable for device implantation.

Soft lithography

Soft lithography can be favorable when the architecture of the desired structure cannot be appropriately constructed due to Computer-aided Design (CAD) constraints; it occurs using

elastomeric stamps and molding procedures (Wu). A study developed branching channels using Lindenmayer Systems (L-Systems)(Wu). L-Systems are an algorithm for producing successively repeating parts of a simple initial object (Prusinkiewicz). Branching channels provide oxygen and nutrients for the cells (Wu). After the L-System based branching design was completed, 3D tissue scaffolds were fabricated layer-by-layer using “UV-Maskless Photolithography” using polyethylene (glycol) Diacrylate (PEGDA)(Prusinkiewicz, Wu). Natural templates have also been used to create branching synthetic vascular systems (Wu). In one study, plant leaves were used as templates for soft lithography (Wu).

Self-assembly

Tissue engineering by self-assembly (TESA) uses cells to fabricate structures that can be applied in vivo without immune rejection. Many diverse approaches can be used for TESA such as cells, peptides, DNA, and chemical interactions (Peck, Zorlutuna, Sanz).. TESA can be performed both in- vivo and in-vitro. The cells synthesize natural extracellular matrix, the backbone of the self-assembled tissues (Peck). Many cell types have been shown to contribute to self-assembly of tissues such as mesenchymal cells (which can differentiate into smooth muscle cells), epithelial cells, vascular cells, endothelial cells, and human skin fibroblasts (Peck, Jakab). TESA can occur layer by layer and create vasculature through a range of methods. For example, substrate-directed self-assembly of vascular cells was accomplished by exploiting vascular mesenchymal cells' (VMCs) ability to form radial structures or concentric rings using the chirality of VMCs, VMCs' spontaneous aggregation, and amplification of the differences in distribution of initial cell plating (Chen). Pluripotent stem cells can also create vascular structures in a synthetic matrix and the networks can integrate with the host's vasculature (Kusuma).

Hydrogels

Hydrogels are useful tools for synthetic vascularization due to their versatility to form unique shapes, high water content, and biocompatible properties. A highly porous synthetic or natural ECM or scaffold is required to accommodate mammalian cells, direct their growth, and regeneration of the tissue in three dimensions. A hydrogel's first generation comprises a wide range of crosslinking procedures involving the chemical modifications of a monomer or polymer with an initiator. The general aim was to develop material with high swelling, good mechanical properties and relatively simple rationale. Then, starting in the seventies, a different concept of hydrogel grew in importance: a second generation of materials capable of a response to specific stimuli, such as variations in temperature, pH or concentration of specific molecules in solution. These specific stimuli can be exploited to trigger specific events, for example the polymerization of the material, a drug delivery, or an in situ pore formation (Rahman). Finally, a third generation of hydrogels is focusing on the investigation and development of stereo complexed material hydrogels crosslinked by other physical interactions. There is an increasing interest in the development of "smart hydrogels", polymeric matrices with a wide spectrum of tunable properties and trigger stimuli. These hydrogels could possibly become fourth generation hydrogels and be applied to an unfathomable range of applications (Jiang). Examples of promising hydrogels include Alginate, *Poly(ethylene glycol)*, Collagen, and Gelatin.

Alginate

Alginate is easy to obtain, is nonimmunogenic, and gels in the presence of calcium to form a hydrogel with a high water content that allows good exchange of waste products and nutrients (Hunt). Covalently or physically cross linked alginate hydrogels are one of many natural polymers used as delivery vehicles of vascular growth factors to induce and increase

neovascularization of ischemic tissues. In all cases studied, alginate hydrogels have presented good degradation and long term growth factor release which consequently increased neovascularization and therefore is recommended for cell/therapeutic delivery and vascular disease treatment (Lee, Nguyen, DeVolder). Ca-alginate gels have been shown to not provoke adverse responses such as thrombosis or fibrosis, even when providing localized release of VEGF (Hunt). Ca-alginate gels have also been shown to replace a role of the extracellular matrix (ECM) by providing a temporary physical support to the damaged tissue (Hunt).

Poly(ethylene glycol)

Poly(ethylene glycol) (PEG) based hydrogels are a versatile option for tissue engineering due to their high hydrophilicity and lack of inherent protein binding sites (Mahadevaiah). PEG hydrogels can be used for cell culture, controlled release of biomolecules, and have been successfully shown as a hospitable environment to promote tissue regeneration. Photopolymerizable hydrogels such as PEG hydrogels allow in vivo formation of complex configurations due to greater control over their polymerization and gelation processes (Bryant). PEG gels can also be formed with varying mechanical and biochemical properties which allows for a wider range mimetic tissue types since these properties influence the growth of seeded cells, other biological materials (Bryant, Gunn). Vascular networks in PEG hydrogels can be induced by decreasing matrix modulus (Mahadevaiah). Larger amounts of ammonia cross linker used in the polymerization of an 8 arm PEG macromeres, lead to higher crosslinking density, an increased surface elasticity and generally smoother surface morphologies (Hutanu). The swelling of these hydrogels were shown to increase with increasing PEG-acrylate and dithiol molecular weights (Yom-Tov). PEG hydrogels incorporating Pluronic F-127 micelles have been shown to form nanostructured materials that facilitate cell spreading (McGann). Protein-PEG

hydrogels comprising other proteins, such as collagen or fibrinogen, have been shown to improve biocompatibility and cell–matrix interaction (McGann). A single resilin-like polypeptide, RLP24, can be added to PEG hydrogels and creates a degradable, elastic, rapidly forming and rubber-like hydrogel; that would be useful for mechanically demanding tissue engineering applications, such as cardiovascular tissues (McGann). Hybrid SCS/PEG hydrogels as injectable scaffolds can be used in soft tissue engineering applications (Tan). Chitosan with PEG hydrogels can allow for water molecules to incorporate more thoroughly through the material (Buranachai).

Collagen

Hydrogels produced from type I collagen have been shown to form millimeter-long perfusable capillaries within 3 days (Linville). PEG-collagen hydrogels were also a tool for vascularization and observed to support ECs well, as the result of Human umbilical vein endothelial cell culture, vessel network formed reached $3 \times 10^4 \mu\text{m}$. MSC delivery by collagen hydrogel showed potential for wound healing. The wounded area treated with the hydrogel healed after 11 days with only slight variability in others which healed in 13 and 15 days. Micro vessels formed per hpt with the hydrogel treatment was 8 according to (Rustad) . (Wang, F) synthesized collagen based injectable hydrogel for IGF and MSC encapsulation undergoing gelation in 6s at physiological temperature, the hydrogel compressive modulus was found to be 63 to 120 KPa, thus the hydrogel serves as a promising device for cell delivery.

Gelatin

Previous works showed that addition of gelatin to dextran photocrosslinkable hydrogel for SMC encapsulation, can modify gel's cell adhesion and compressive stress as they are 37.42 and 51.94 for elastic and compressive modulus respectively, release occurs after 3 days along

with gel degradation (Anderson-Shaw). UV cross linkable pure gelatin was reported for MSC and ECFC entrapment. UV light exposure found to have no adverse impact on cell viability, but the elastic and viscous modulus are controllable with the alteration of light exposure up to 120 seconds. Vessels formed in unit mm^2 increases when the exposure time is short (Xu). Gelatin hydrogels can also provide slower release for growth factors namely VEGF and TGF β 1; release time was increased to 2 weeks, and injection of both platelet rich plasma and gel led to capillary vessel formation of 844 capillaries per mm^2 (Witzenbicher). Similarly, the capillary density of Endothelial progenitor cells and VEGF filled hydrogels had an arterial density of 12.5 (count per mm^2) (Pérez-Ruiz). A microfabrication technique (soft lithography) applied on gelatin methacrylate for entrapment of HUVEC, VEGF and bFGF, provided a good 3D environment for tubulogenesis; in consequence visible vessel formation was observed (Amini). Physically formed gelatin hydrogels including HUVEC (Zhu) and HUVEC-fibroblast (Tedesco) both resulted in vessel formation of 200-300 vessels for the first and tube length of 150 μm . A bFGF, VEGF and HGF filled Ozeki's gel resulted in formation of 3 to 9 blood vessels per mm^2 . Controlled release of multiple growth factors including PDGF-BB, VEGF, EGF, TGF β and bFGF from hydrogel effectively increased neovascularization by ratio of 3 to 3.5 (Raff). A combination of hydrogel and electrospun scaffolding with VEGF and BMP-2 reported double the amount of vessel volume compared to scaffold alone (Yahia). Preparation of cationized gelatin with HUVECs within the gel, led to improvement of hydrogel's mechanical properties up to 0.15-2.5 KPa (Mahadevaiah).

Methods

Scaffold Creation

The 3d scaffold mold was designed in SolidWorks 2014 and built in resin.

Hydroxyapatite (HA) was synthesized according to Cüneyt Tas process A (Cüneyt Tas). Then it was calcinated using a furnace a 700 C for 6 hours (the HA was heated stepwise following 250°C for 10 min, 450°C for 20 min, and 700°C for 6 hours). The calcination process helps control the size and shape of the resulting particles and can lead to TCP content with temperature increasing temperatures (Guo, Lazar). The hydroxyapatite was phosphorylated with phosphoric acid solution. The resulting paste was press-injected in the mold, dehydrated, and the mold was burned off at 400°C for 120 min. The scaffold was imaged using a VEGA 3 Tescan scanning electron microscope (SEM) where samples were prepared and placed into a deaton vacumm desk ii for gold coating.

Media Degradation Test

Half of the scaffold samples were submerged in 1.5 mL of SBF (Simulated Body Fluid) and the other half were submerged in 1.5 mL of PBS (Phosphate Buffered Saline) plates. At 5 day intervals 2 SBF and 2 PBS samples had the media removed by aspiration and were washed with DI water and left to dry for 6 hours prior to weighing.

Sterilization and Macrophage Degradation testing

The scaffold portions were sterilized with amphotericin-B, streptomycin, and penicillin for 45 min. The sterilization media was removed by aspiration, washed once with PBS to remove the antibiotics and the scaffold portions dried for 6 hours. 150µL of cell suspension was added to each dish containing a scaffold portion. Phorbol myristate acetate (PMA) was added to half of the samples to induce the monocytes to differentiate into macrophages and have an increased amount of macrophages compared to monocytes. To the other half of the samples 1.5 mL of PMA solution and 1.5 mL basal media (90% IMDM and 10% FBS) was added. Cells were incubated at 37 degrees C over the 15 day period. At 5 day intervals the samples were removed

from cell suspension by aspiration. The scaffold in the plate was washed with DI water to remove cell mass and left to dry for 6 hours prior to weighing (to measure only the change in the amount of scaffold and not water weight or cell mass). The cell proliferation level can be measured by culturing and incubating cells, adding 20uL/well of MTS Reagent and incubating for 2 hours at 37°C, placing the well plate on a shaker briefly and measuring the absorbance of untreated cell culture vs treated cell cultures at 490nm (seeded at similar cell densities to only measure the variable of interest) using a plate reader.

Compression disks

50% HA to TCP with a 1:2 ratio of 50% phosphoric acid to make paste and 25% HA to TCP with a 1:2 ratio of 50% phosphoric acid to make paste was heated in a furnace to 100°C for 30 min. Each disk was molded to be 1 cm by 0.5 cm. This was to examine if the amount of HA compared to the amount of TCP had an effect on mechanical strength.

Results

Commercial Hydroxyapatite (HA) comparison with synthesized HA

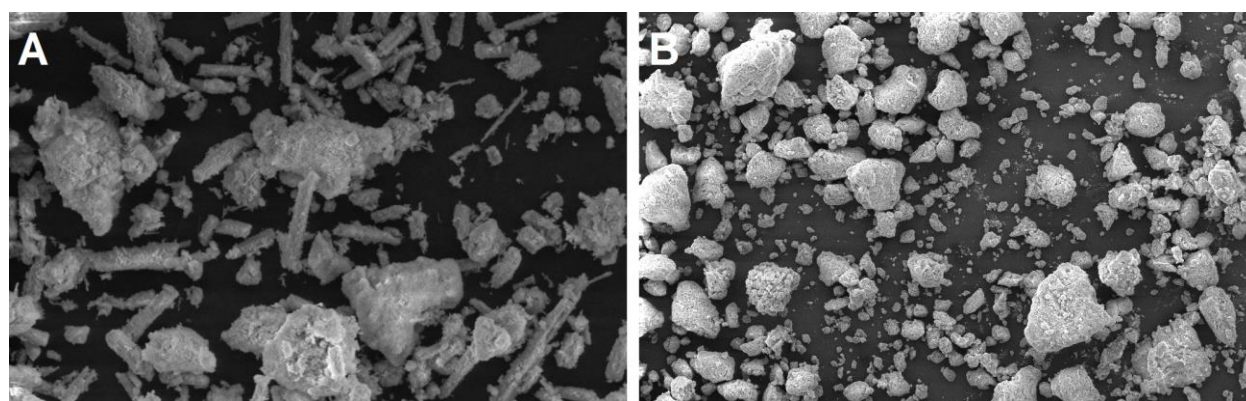


Figure 2. Synthesized HA (A) compared to commercial HA (B) SEM images. HA as can have different Ca to P ratios which can affect both its chemical and mechanical properties.

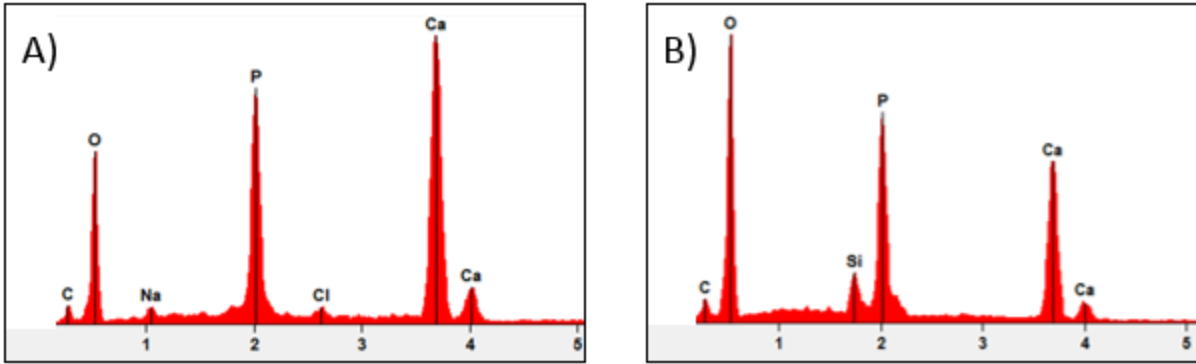


Figure 3. Energy-disperse X-ray spectroscopy (EDX) Spectra of synthesized HA (A) compared to commercial HA (B)

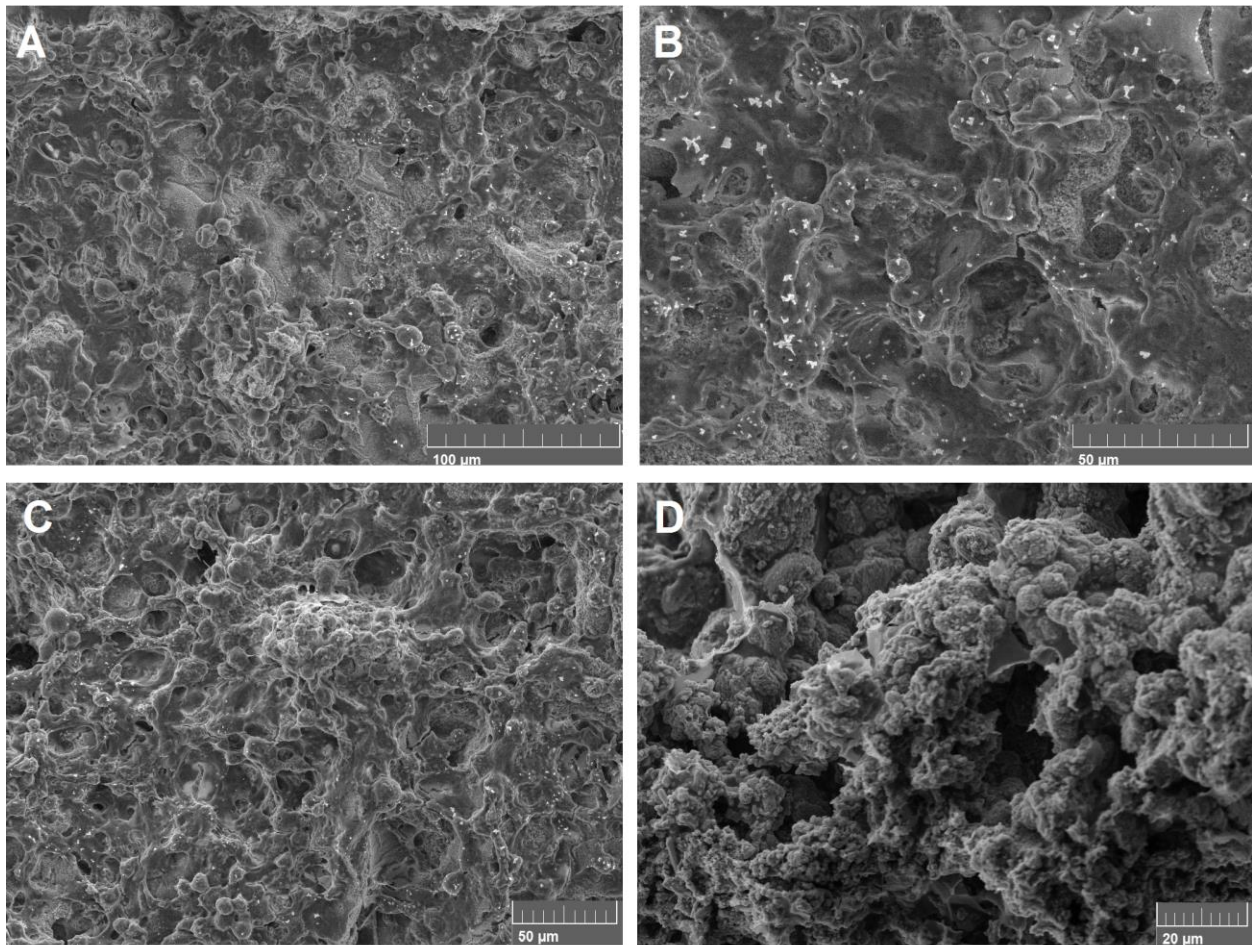


Figure 4. SEM images of scaffold after cellular seeding. After 10 days of incubation with the scaffold SEM images were taken to see if macrophages can adhere to the calcium phosphate scaffold. Initial monocyte adhesion is mediated by $\beta 2$ integrins, and $\beta 1$ integrins play a role in adhesion during the induction of macrophage fusion by IL-4, and the material modulus plays a role in macrophage adhesion and behavior (Irwin, McNally). Molded microchannels were 500 μm , but after imaging there were also visible micropores that were as small as 20 μm or smaller (D).

Pore Size

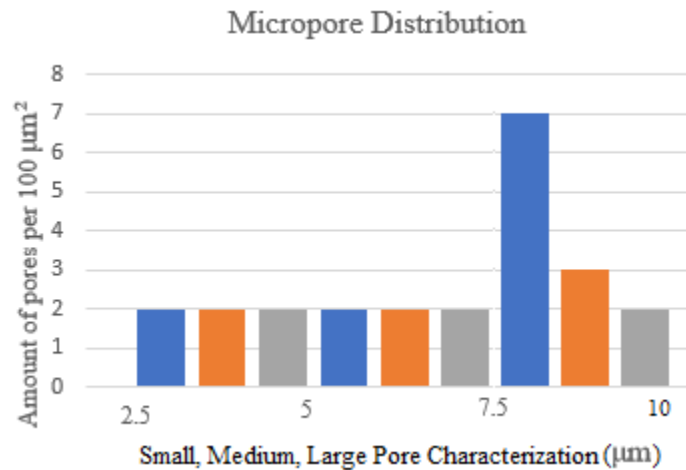


Figure 5. In a 100 micrometer squared field area there were on average 2 large pores (10.0-7.5 micrometers wide), 2 medium pores (7.5-5.0 micrometers wide), and 6 small pores (5.0-2.5 micrometers wide) and were approximately circular or oval shaped.

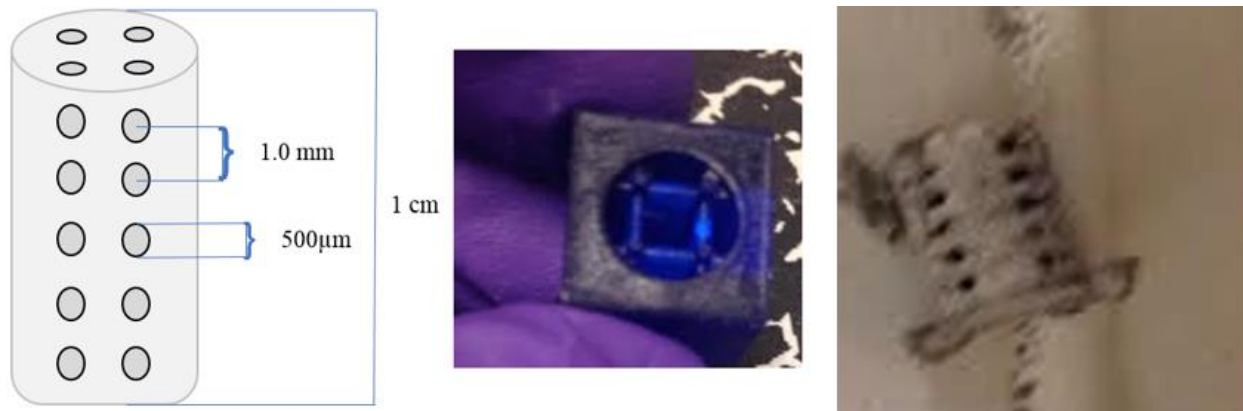


Figure 6. For each artificial osteon core, paste was press injected into the 3D printed mold and the mold was burned off in a furnace to produce a scaffold with fourteen 500 μm macropores to guide the formation of vascular networks.

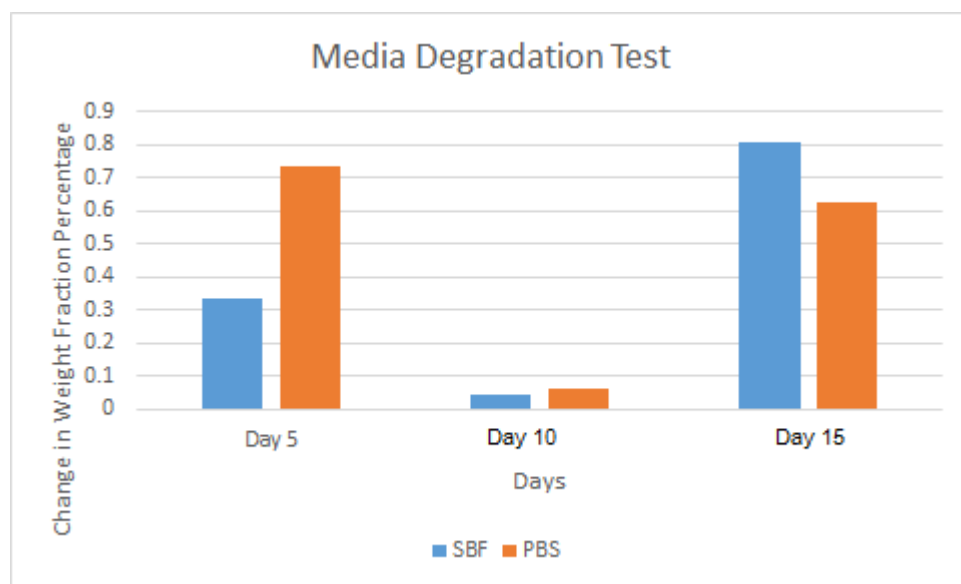


Figure 7. Scaffold degradation in Simulated Body Fluid and Phosphate-buffered saline. This test served as a control comparison test in order to see how the scaffolds withstand in non-cellular environments.

Cellular Degradation Test

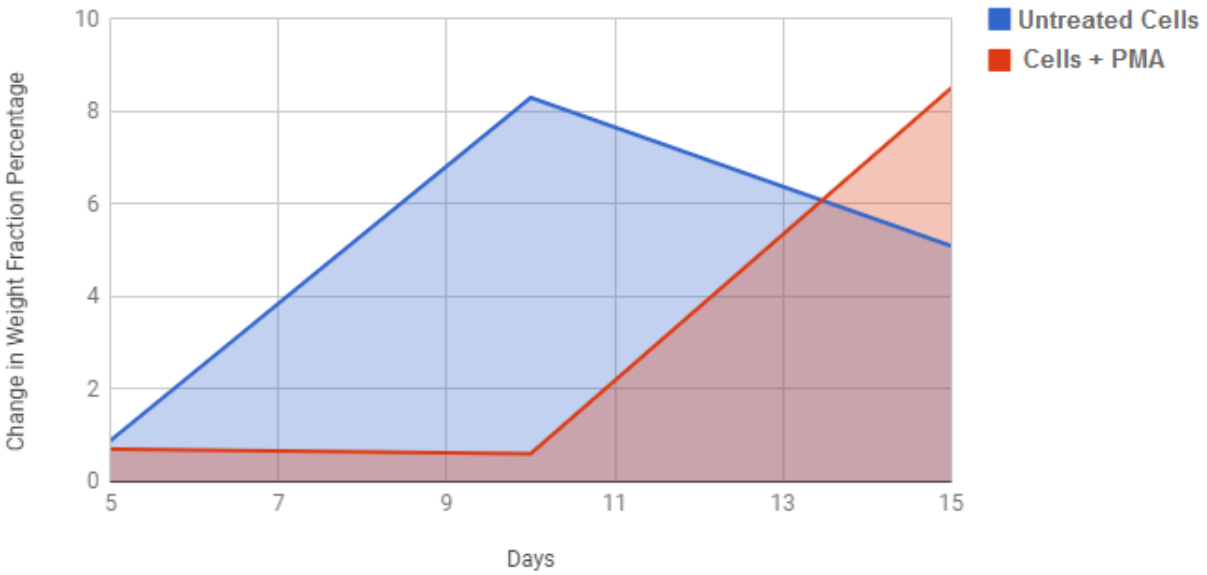
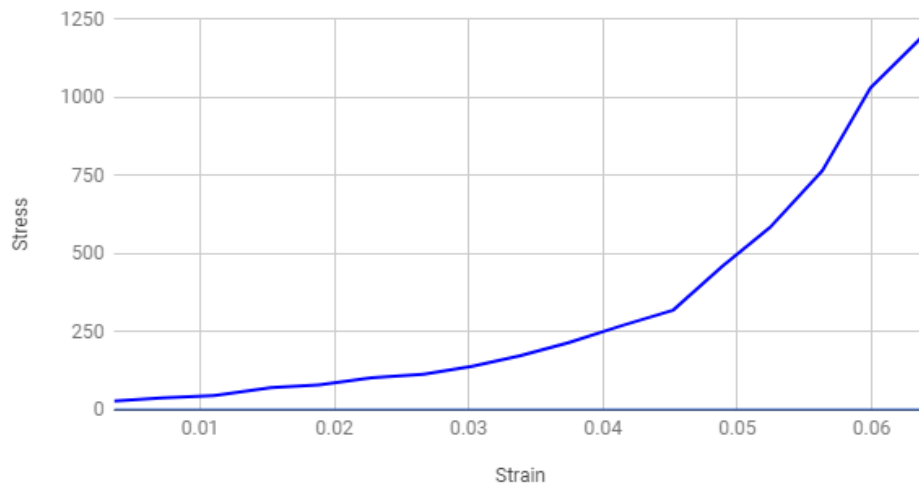
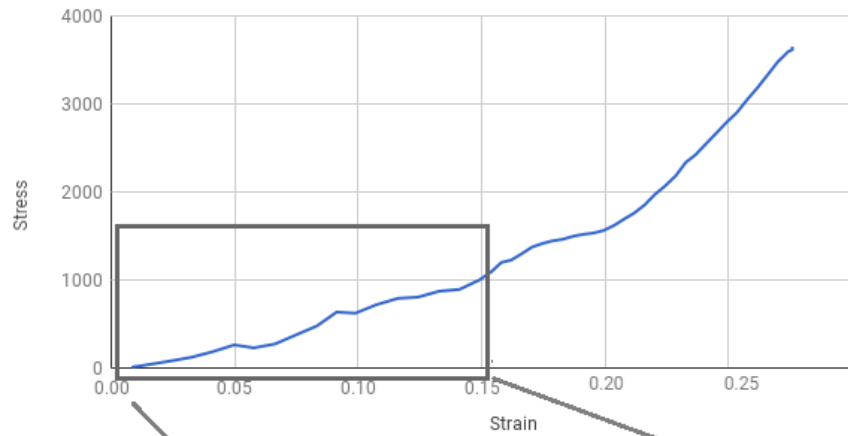


Figure 8. Cellular Degradation test. The average change over five days for cells without PMA was 3.27%. The average change over five days for cells with PMA was 4.77%. $y = 0.0184x^2$ was the line that most closely predicted the data for the cells + PMA (macrophages) (other than a 3rd order polynomial which would of had an $R=1$). $y = 0.0701x^{1.75}$ was the line that most closely predicted the data for the untreated cells.

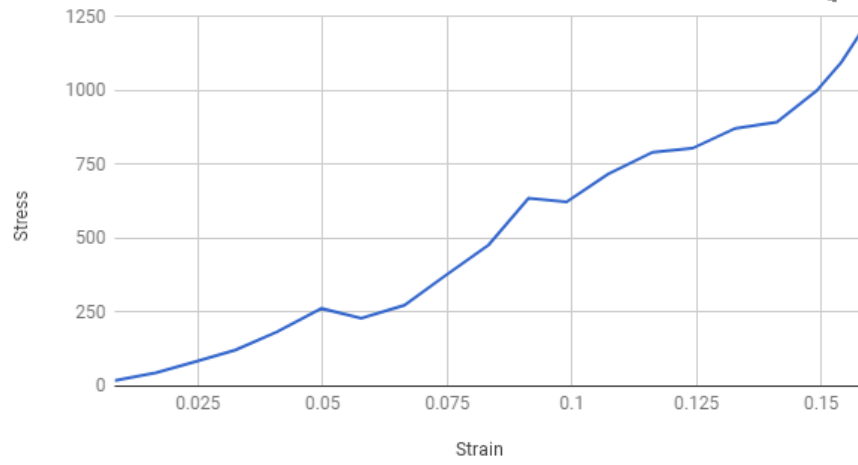
50% HA-TCP



25% HA-TCP



25% HA-TCP



50% HA-TCP

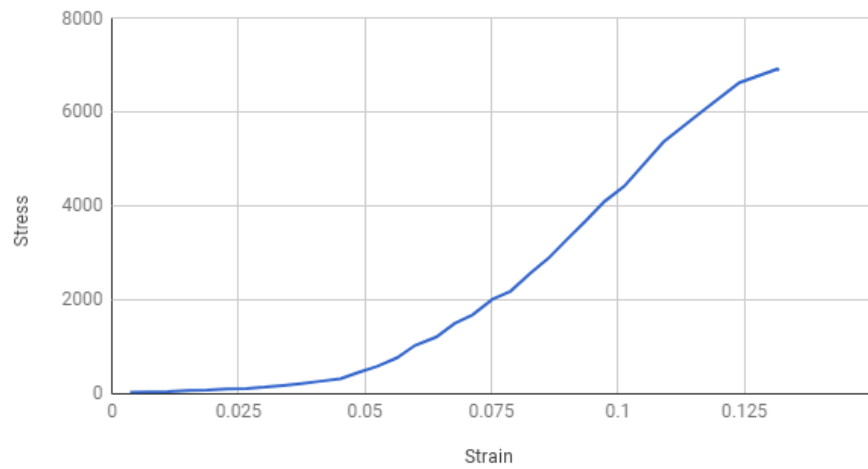


Figure 9. Mechanical test of 25% and 50% HA-TCP paste disks using compression with a parallel plate for soft solids to determine stress and strain. This followed standard patterns of brittle materials with a sudden break point. Compared over the same range of strain the 50% HA/TCP could withstand more stress but had an earlier break point.

Discussion

Osteopathic surgeons cite that one of the main difficulties with bone substitutes is tissue rejection or infection. This calls for biocompatible and fast healing bone substitutes. A problem with current bone filler pastes is that they can only be used for non-load bearing applications such as dental and orthopaedic cranio-facial applications as approved by the FDA. Using the methods presented, the osteogenerative scaffold could expand the range of appropriate tissue repairs being conducted. A trend to solve this problem is called the diamond concept. This concept pushes research to enhance bone fracture healing by combining osteogenic cells, osteoconductive scaffolds, growth factors, and the optimal mechanical environment such as porosity (Giannoudis).

Since multiple trials resulted in ash or resin residue being left along the scaffold, a post curing step was added after injecting the paste into the mold. The cube molds were placed under UV light for 20 min. This reduced the amount of resin residue left in the pores. Different solvents (Dichloromethane , Dimethylformamide , Dimethyl sulfoxide, Isobutyl Alcohol, Isopropyl alcohol) were also tested to see if they could degrade the resin by a possible solvolysis mechanism and leave the scaffold; the DMF started to produce the most amount of visible cracks in the resin after 20 days of soaking in 20mL of solvent. Additionally the scaffold geometry would become altered by forming a bubble along the top of the scaffold, so different dehydration schedules were tested. 80°C for 24 hrs, 80°C for 2 hrs followed by 95°C for 2 hrs followed by

110°C for 2 hrs, and 110°C for 4 hrs. The six hour stepwise schedule was found to be optimal. Acidity was also changed from 20% phosphoric acid to 50% phosphoric acid due to the formation of bubbles if too much water content was in the sample and caused a loss of scaffold shape; increased acid concentration reduced the amount of phosphoric acid necessary to make paste, and reduced the phosphoric acid to HA-TCP ratio to 2:1. Uncompressed molds do not hold their shape so a plunger was created to hand press the paste to create the scaffold. The CAD mold creation and press-inject technique was beneficial because this could be applied to industrial applications and mass production, but to increase the range of defect geometries that can be repaired quickly without altering the mold, the technique could be altered as shown in the figure.

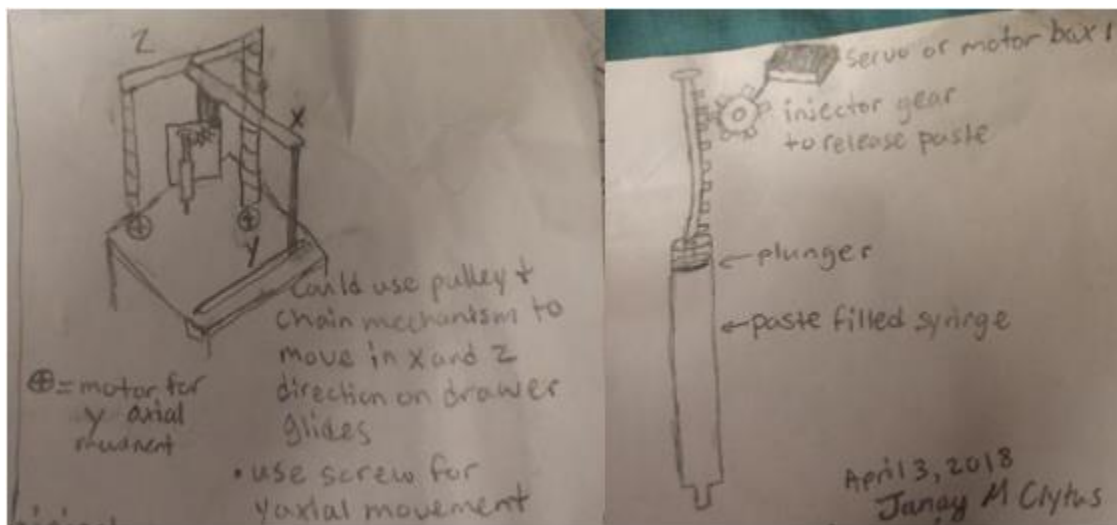


Figure 10. Alternative fabrication technique. A cube pro or similar FDM printer could have the injector head modified, so the programming language would be the same and the same files would code for the same movements; the servo or motor box 1 speed would have to be programmed to run at a similar extrusion speed as the cube pro (or make HA filament spools, but this could be difficult due to the nature of the paste, a thin, meltable, adhesive cylindrical

structure bag would have to surround each loop of paste on the filament spool, and the paste would have to be kept away from heat (due to the paste becoming brittle) until the 3D printed final form is done then the machine could heat up.

The schedule that produced the least amount of a bubble during water removal was 80°C for 2 hrs followed by 95°C for 2 hrs followed by 110°C for 2 hrs. ABS plastic molds were also tested. ABS is amorphous and has no true melting temperature. 400°C did not remove all of the plastic, but 60°C (30 min) then 500°C (2 hrs) worked to remove most of the plastic but left resin in the channels. Other ABS burnoff replicates, turned the scaffold to ash. ABS plastic did not burnoff as well as the castable resin.

For a viable scaffold, tests were conducted to insure that the scaffold could withstand degradation for a long enough amount of time for the body to properly heal. The first 7 to 10 days of fracture healing involves a process called chondrogenesis, which leads to cartilage formation adjacent to the fracture site and the formation of bone directly from osteoprogenitor cells under the periosteum. During this time an inflammatory response takes place at the fracture site, as demonstrated by the invasion of macrophages, polymorphonuclear leukocytes, and lymphocytes, which secrete proinflammatory cytokines (Einhorn). The scaffold was seeded with macrophages in order to test if the scaffold could withstand acidic cellular secretions activated in the immune response that degrade the scaffold for a long enough period for the implant to integrate with the body's natural tissue and cells to differentiate from collagen to bone. Under typical normal biological conditions, many skeletal fractures heal in the first 6 to 8 weeks (Gómez-Barrena). In patients without osteoporosis it took 16.19 ± 5.07 weeks for femoral fractures to heal, and patients with osteoporosis took 19.38 ± 5.9 weeks for femoral fractures to heal (Nikolaou). There was an average change over five days of 4.77%. For 100% degradation

this would be estimated to take approximately 104 days. For patients without osteoporosis it took 113.33 days for the fracture to heal, so the scaffold parallels the time course needed for healing.

Additionally, the scaffold also contains phosphate. The advantage of phosphate is that it aids in inducing bone regeneration (phosphorylated protein in phosphate activated cell signaling).

Phosphate may have a connection with vitamin D metabolism. Phosphate is necessary for proper mineralization of bone as a constituent of hydroxyapatite crystals (Fukumoto). Phosphorylated proteins like osteopontin and dentin matrix protein 1 (DMP1) have been shown to regulate bone mineralization. Phosphate is also a constituent of biomembranes and nucleic acids (Fukumoto).

Furthermore, phosphorylated metabolites such as adenosine triphosphate, 2,3-diphosphoglycerate, glucose-6-phosphate and phosphorylated proteins are necessary for energy metabolism, differentiation, proliferation and specific function of differentiated cells.

Hypophosphatemia can lead to muscle weakness, rhabdomyolysis, consciousness disturbance and rickets/osteomalacia characterized by impaired mineralization of the bone matrix (Fukumoto). Cell migration control is a main component behind creating the radial structures needed for vascular structures. Moreover, co-culturing of ECFCs and MSCs is significant because vasculogenesis is necessary to bring nutrients to the cells so they don't die off prior to differentiating into bone. This is important also because immune cells use the bloodstream to travel to the site of infection if inflammation occurs in order to have proper healing of the tissue.

Conclusion

A hydroxyapatite scaffold can be fabricated through the process of rapid prototyping. The composition of synthesized HA was more similar to natural HA than the commercial HA with a higher ratio of calcium and phosphate than oxygen. The scaffolds can withstand immune degradation, so the scaffold will survive long enough to successfully promote healing of the

bone. The scaffold also allows for cell adhesion and includes microfluidic channels to induce proper overall shape of regenerated tissue vascularization. Synthesized HA had a more crystalline structure than the commercial HA (Sigma Aldrich St Louis, MO), but commercial HA was used for further testing to have more consistency in HA composition between tests. More replicates can be performed to further back the data. The scaffold must be able to be applicable in load bearing applications if used to help repair compact bone such as in the femur. Tissue engineering might be far from order ready patient specific tissues, but the field is advancing in creating tissues that are very similar to their natural counterparts in both function and composition. For tissue biocompatibility, vascularization is a must. The process of vascularization could possibly be catalyzed in the future for a shorter recovery time in patients. As mentioned previously, smart hydrogels and scaffolding will create an extensive range of variable properties that could enhance future vascularization in synthetic tissues. Scaffolding itself can enhance and guide bone repair, by incorporating growth factors and allowing for proper nutrient exchange (Rahman).

Since the cells adhere to the scaffold, this will allow for guidance of bone repair. Future work can focus on stem cells. The ratio of HA to TCP can be produced at the level where ECFC cell viability was experimentally shown to be at the highest level compared to other ratios, meaning that this recipe is optimized for biocompatibility. Also there could be incorporation of Keratin. Keratin hydrogels support the sustained release of bioactive ciprofloxacin, which could reduce the chance of infection after the surgical procedure. Osteogenerative scaffolding could later model spongy bone and be used in spinal implants. A hydrogel and HA/TCP scaffold middle disk insert structure mimics the spinal vertebrae and intervertebral disc structure. Furthermore, a study discussed co-culturing vascular mesenchymal cells with non-pattern-

forming endothelial cells (ECs) and this approach allowed for cellular self-organization and formed a coherent radial or ring pattern mimicking the cross-sectional structure of liver lobules or osteons (Chen). The bone matrix also contains glycoproteins. Glycoproteins that are highly phosphorylated are called phosphoproteins (osteonectin, osteopontin, bone sialoprotein, dentin matrix protein 1, matrix extracellular phosphoglycoprotein and acidic glycoprotein-75). The glycoproteins are buried in the inorganic elements of the bone matrix and are believed to affect bone calcification (Ascenzi). Glycoproteins may influence osteogenesis.

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